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Agonist and Antagonist Effects of Diadenosine Tetraphosphate, a Platelet Dense Granule Constituent, on Platelet $P2Y_1$, $P2Y_{12}$ and $P2X_1$ Receptors

Hung Chang[a,b], **Ivan B. Yanachkov**[c], **Alan D. Michelson**[a,d], **YouFu Li**[a], **M.R. Barnard**[a,d], **George E. Wright**[c], and **Andrew L. Frelinger III**[a,d,*]

[a]Center for Platelet Function Studies, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA

[b]Hematology Division, Chang Gung Memorial Hospital, Chang Gung University, Taipei, Taiwan

[c]GLSynthesis Inc., Worcester, MA

[d]Center for Platelet Research Studies, Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, MA

Abstract

Introduction—Diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) is stored in platelet dense granules, but its effects on platelet function are not well understood.

Methods and Results—We examined the effects of Ap_4A on platelet purinergic receptors $P2Y_1$, $P2Y_{12}$ and $P2X_1$. Flow cytometry was used to measure the effects of Ap_4A in the presence or absence of ADP on: a) $P2Y_{12}$-mediated decrease in intraplatelet phosphorylated vasodilator stimulated phosphoprotein (VASP), b) $P2Y_1$-mediated increase in platelet cytosolic Ca^{2+}, and c) $P2X_1$-mediated intraplatelet entry of extracellular Ca^{2+}. ADP-stimulated platelet shape change ($P2Y_1$-mediated) and aggregation ($P2Y_1$- and $P2Y_{12}$-mediated) were measured optically. Ap_4A inhibited 3 μM ADP-induced: a) platelet aggregation (IC_{50} 9.8 ± 2.8 μM), b) $P2Y_1$-mediated shape change, c) $P2Y_1$-mediated increase in platelet cytosolic Ca^{2+} (IC_{50} 40.8 ± 12.3 μM), and d) $P2Y_{12}$-mediated decrease in VASP phosphorylation (IC_{50} >250 μM). In the absence of added ADP, Ap_4A had agonist effects on platelet $P2X_1$ and $P2Y_{12}$, but not $P2Y_1$, receptors.

Conclusion—Ap_4A, a constituent of platelet dense granules, is a) an antagonist of platelet $P2Y_1$ and $P2Y_{12}$ receptors, where it inhibits the effects of ADP, and b) an agonist of platelet $P2X_1$ and $P2Y_{12}$ receptors.

***Corresponding author:** Andrew L. Frelinger, PhD, Children's Hospital Boston, 300 Longwood Avenue, Karp 07212, Boston, MA 02115-5737. andrew.frelinger@childrens.harvard.edu Phone: (617) 919-2537. Fax: (617) 730 4632.

Conflict of Interests Statement

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Keywords

ADP; diadenosine tetraphosphate; platelets; purinergic receptors; platelet aggregation

Introduction

Platelets express three purinergic receptors, $P2X_1$, $P2Y_1$ and $P2Y_{12}$ (Fig. 1).[1,2] $P2X_1$ receptors are activated by adenosine 5'-triphosphate (ATP) while $P2Y_1$ and $P2Y_{12}$ receptors are both activated by adenosine 5'-diphosphate (ADP) [1,2]. The $P2X_1$ receptor is a ligand-gated ion channel which upon activation triggers fast influx of extracellular Ca^{2+} into the cytoplasm and transient platelet shape change (Fig. 1) [1–4]. $P2Y_1$ and $P2Y_{12}$ are G-protein coupled receptors, $P2Y_1$ being coupled to G_q and $P2Y_{12}$ to G_i[1,2]. Upon activation, $P2Y_1$ triggers Ca^{2+} mobilization from the platelet dense tubular system, shape change, and reversible platelet aggregation (Fig. 1) [1,2]. Activation of $P2Y_{12}$ leads to inhibition of the adenylyl cyclase-dependent production of cytoplasmic cyclic adenosine 5'-monophosphate (cAMP) and propagation of stable platelet aggregation [1,2]. cAMP activates protein kinase A which then phosphorylates vasodilator stimulated protein (VASP) [2,5], a modulator of platelet cytosolic proteins (Fig. 1). Both $P2Y_1$ and $P2Y_{12}$ play major roles in the amplification and stabilization of platelet activation. The exact physiological role of $P2X_1$ is less clear, but it plays a role in the enhancement of the effect of low levels of primary platelet activators and in high shear stress activation [1,2].

Diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) and other diadenosine polyphosphates are naturally occurring compounds that are ubiquitous in mammalian tissues[6], including human platelets[7,8]. They may serve as neurotransmitters[9] and modulators of vascular tone [10].There is growing evidence that Ap_4A plays a role in systemic diseases such as diabetes mellitus and hypertension [11,12]. In platelets, Ap_4A is stored in dense granules, and is therefore released along with ADP and ATP upon platelet activation[8,13]. Ap_4A and its analogs are known to inhibit ADP-induced platelet activation[14,15]. Ap_4A analogs inhibit the ADP-induced platelet release reaction, calcium mobilization, thromboxane production and platelet factor 3 activities[14]. However, these studies[14,15] were performed before all three platelet purinergic receptors were cloned and their functions characterized. Therefore, the mechanism by which Ap_4A inhibits ADP-induced platelet activation and its possible effects on $P2Y_1$ and $P2Y_{12}$ are unknown. Diadenosine polyphosphates are potent agonists of P2X receptors expressed on a variety of human and rat cell types[16,17]. Although human platelets express $P2X_1$ receptors[18–20], whether Ap_4A is an agonist via platelet $P2X_1$ is unknown. The goal of the present study was, therefore, to elucidate the effects of Ap_4A on signaling through $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors on human platelets. We demonstrate that Ap_4A, a known constituent of platelet dense granules, is: a) an antagonist of platelet $P2Y_1$ and $P2Y_{12}$ receptors, where it inhibits the effects of the agonist ADP, b) an agonist of $P2X_1$ receptors, and c) a partial agonist of $P2Y_{12}$ receptors.

Materials and Methods

Chemicals and reagents

Ap_4A was synthesized by a novel method (to be published) and was >98% pure by reverse phase HPLC. MRS2179, MRS2159, probenecid, adenosine 5'-(β,γ-methylene)triphosphate (β,γ-CH_2-ATP) and apyrase (grade VII) were purchased from Sigma-Aldrich (St. Louis, MO). D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was purchased from Calbiochem (EMD Biosciences, La Jolla, CA). FLUO-4 was from Invitrogen (Carlsbad, CA),ADP was from Bio/Data (Horsham, PA), CD41-phycoerythrin (PE)-Cy5 was from Beckman Coulter (Fullerton, CA), and AR-C69931 was from AstraZeneca (Charnwood, UK).

Blood collection and sample preparation

Human blood samples were taken from healthy volunteer donors who had been free from aspirin or other non-steroidal anti-inflammatory drugs for more than 7 days. IRB-approved written informed consent was obtained before blood collection. Unless otherwise specified, blood was drawn from antecubital veins into 3.2% sodium citrate tubes. Whole blood was used in VASP phosphorylation and $P2Y_1$ cytosolic Ca^{2+} assays. For platelet aggregation tests, the blood was centrifuged at 110 g for 12 minutes and platelet-rich plasma (PRP) was immediately removed. The remaining samples were further centrifuged at 1650 g for 10 minutes to obtain platelet-poor plasma (PPP), which was used as a reference. For assays of platelet $P2X_1$ receptor functions, whole blood was drawn into tubes containing PPACK (0.3 mM, final concentration) and apyrase (1.8 μM final concentration). The samples were then centrifuged and processed to retrieve PRP and PPP.

ADP-induced platelet aggregation and shape change

Light transmission platelet aggregation was performed as previously described[21]. ADP (3 μM) in the presence or absence of various concentrations of Ap_4A was added to PRP, and the aggregation response was recorded for a total of six minutes in a Chrono-log® aggregometer running the Aggro/Link software (Chrono-log®, Havertown, PA).

For observation of ADP-induced platelet shape change, EDTA (final concentration 10 mM) was mixed with PRP to allow ADP-induced shape change but to avoid platelet-platelet aggregation. After obtaining a stable baseline tracing in the aggregometer ADP (3 μM), in the presence or absence of various concentrations of Ap_4A, was added to induce platelet shape change, and the tracing recorded for a total of 4 minutes.

Platelet aggregation measured by microplate reader

The 96-well microplate method for the detection of platelet aggregation is a simple, rapid, low volume method to simultaneously measure platelet aggregation of multiple samples, thereby avoiding the variable of platelet aging[22,23]. In brief, ADP (5 μL, final concentration 3 μM) and Ap_4A (5 μL, at various concentrations) were added to each well of a flat bottom 96-well Immulon microplate (Thermo Fisher Scientific, Waltham, MA). PRP (90 μL) was then added to each well with a multi-channel pipette, and light transmission was recorded with a Molecular Devices microplate reader, running SOFTmax Pro 4.0 software at 580 nm wavelength under the kinetic mode, with intermittent shaking and readings every 11 seconds at 37°C. Results of platelet aggregation measured by the microplate method have been demonstrated to be comparable to results of conventional platelet aggregation[22,23]. Similarly, we observed comparable Ap_4A inhibition of ADP-stimulated platelet aggregation by these two methods in the present study (Figure 2A and 2B). Since the microplate method avoids possible aging artifacts, results from this method were used to calculate the IC_{50} for Ap_4A.

$P2Y_{12}$-mediated VASP phosphorylation assay

VASP phosphorylation (Fig. 1) was measured by flow cytometry[5] using a kit (BioCytex, Marseilles, France) essentially according to the manufacturer's recommendations. In brief, Ap_4A or vehicle (2 μL) was added to each set of assay tubes, followed by prostaglandin E_1 (PGE_1, 9 μL) or the same volume of PGE1 plus ADP. Citrated whole blood (10 μL) was then added to each tube, and the samples were incubated for 10 minutes at room temperature. The samples were then fixed, permeabilized, and labeled with a fluorescently conjugated monoclonal antibody (clone 16C2) directed against the serine 239 phosphorylated form of VASP and, as a platelet identifier, CD61. Analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The platelet reactivity index (PRI) was calculated after subtracting background fluorescence, according to the manufacturer's

recommendations using the formula PRI = [($MFI_{(PGE1)}$−$MFI_{(PGE1+ADP)}$)/$MFI_{(PGE1)}$]×100, where MFI is the mean fluorescence intensity.

HEPES-saline buffer was added as a control (i.e. no antagonist, 0% inhibition). The possible agonist property of Ap_4A on $P2Y_{12}$ was tested by adding Ap_4A to the tube incubated with PGE_1 in the absence of ADP.

To evaluate degradation and the possibility of generating biologically active metabolites of Ap_4A in the VASP assay, Ap_4A was incubated with whole blood and the PGE_1 reagent from the VASP kit for 0, 3, 6 and 10 minutes. The mixture was centrifuged at 13,600 g for 1 minute and the supernatant retrieved and stored at −80°C until analysis. Prior to analysis by high performance liquid chromatography (HPLC), proteins were removed by perchloric acid precipitation and the samples centrifuged (13,000 g) and filtered (0.45 μ nylon).

$P2Y_1$-mediated cytosolic calcium increase measured by flow cytometry with the calcium indicator FLUO-4

The ADP-dependent, $P2Y_1$-mediated increase in platelet cytosolic calcium was measured by detecting changes in FLUO-4 fluorescence (Fig. 1), as previously described[24]. In brief, one part of citrated whole blood was added to 9 parts of a loading solution consisting of FLUO-4 (5 μM final concentration), CD41-PE-Cy5 (1:176 dilution in HEPES-saline buffer [10 mM HEPES, 0.15 M NaCl, pH 7.4]) and probenecid (1 mM final concentration), and the mixture was incubated for 30 minutes at room temperature. Fifteen μL of this mixture were added to 525 μL of HEPES-saline buffer, and the mixture analyzed by a FACSCalibur flow cytometer. After obtaining a 30 second baseline recording, the acquisition was paused, and 60 μL of either ADP (3 μM final concentration), Ap_4A at various concentrations, or ADP (3 μM final concentration) plus Ap_4A at various concentrations were quickly added, the sample mixed, and the acquisition resumed (total pause time less than 10 seconds). FLUO-4 fluorescence was monitored for a total of 2 minutes. FLUO-4 fluorescence was plotted vs. time using FlowJo version 7.2.2 (Tree Star, Ashland, OR) software. The mean FLUO-4 fluorescence of the baseline 30 second interval and of 10 second post-stimulant intervals were calculated. The cytosolic Ca^{2+} increase was calculated as the ratio of the maximal post-stimulant FLUO-4 fluorescence to the baseline FLUO-4 fluorescence. The percent inhibition of ADP-induced calcium increase due to the addition of Ap_4A was calculated relative to ADP (3 μM) plus vehicle (HEPES-saline).

$P2X_1$-mediated entry of extracellular calcium measured by flow cytometry with the calcium indicator FLUO-4

$P2X_1$ receptors are ligand-gated ion channels which, when triggered, cause cytosolic Ca^{2+} to increase by allowing entry of extracellular Ca^{2+} (Fig. 1). Measurement of $P2X_1$-mediated entry of extracellular Ca^{2+} with FLUO-4 differed from measurement of $P2Y_1$-mediated increase in cytosolic Ca^{2+} from intracellular stores in that 1) blood was collected in PPACK (0.3 mM) rather than sodium citrate, to preserve physiologic extracellular Ca^{2+} levels, 2) apyrase (1.8 μM final concentration) was added immediately upon blood collection to degrade any ATP present, thereby avoiding $P2X_1$ receptor desensitization.[1,25], 3) prior to loading platelets with FLUO-4, PRP was separated from erythrocytes in order to minimize erythrocyte-derived nucleotides, and 4) FLUO-4-loaded platelets were diluted and analyzed in buffer containing Ca^{2+} (2 mM) rather than in Ca^{2+}-free buffer to provide physiologic levels of extracellular Ca^{2+}. Platelets in apyrase-treated, PPACK-anticoagulated PRP were loaded with FLUO-4 for 30 minutes in the presence of probenecid (1 mM), diluted in HEPES-saline buffer containing 2 mM $CaCl_2$, and then baseline FLUO-4 fluorescence was obtained. $P2X_1$-dependent entry of extracellular Ca^{2+} was demonstrated by increased FLUO-4 fluorescence upon addition of the specific $P2X_1$ receptor agonist β,γ-CH_2-ATP (20 μM), and the absence of this increase when

platelets were resuspended in 1 mM EGTA, HEPES-saline buffer, or prepared without addition of apyrase to preserve $P2X_1$ activity. FLUO-4 fluorescence after addition of various concentrations of Ap_4A to this system was used to evaluate agonist activity on $P2X_1$. In addition, the ability of Ap_4A (50 μM) to block $P2X_1$ activation by β,γ-CH_2-ATP (20 μM) was tested. To ensure that the increase of cytosolic Ca^{2+} in these experiments was not due to spurious $P2Y_1$-mediated Ca^{2+} increase, some experiments were done in the presence of 1 mM EGTA in HEPES-saline buffer, MRS2179 (100 μM, a $P2Y_1$ receptor antagonist),[26,27] or both.

Statistical analysis

The results were analyzed by GraphPad Prism software, version 4.00 for Windows (GraphPad Software, San Diego, CA). All results are expressed as mean ± standard error of the mean (SEM). Student's t-test was used to determine statistical significance when two groups of data were compared. One way ANOVA and Bonferroni's multiple comparison test were used when three or more groups of data were compared.

Results

Inhibition of ADP-induced platelet aggregation

Ap_4A inhibited maximal ADP-induced platelet aggregation and, at a high concentration (100 μM), prevented secondary aggregation and resulted in disaggregation (Fig. 2A). Dose-dependent inhibition of ADP-induced platelet aggregation determined by the 96-well microplate method is shown in Fig. 2B. The IC_{50} was 9.8 ± 2.8 μM.

Agonist and antagonist properties of Ap_4A on $P2Y_{12}$ receptors

The unstimulated baseline VASP phosphorylation MFI was obtained by treating platelets with PGE1 in all experiments (Fig. 3). As expected, the addition of 3 μM ADP reduced VASP phosphorylation (Fig. 3, open bar). Ap_4A was added to attenuate this ADP effect as a potential antagonist (in comparison with PGE_1+ADP) or to reduce VASP phosphorylation by itself as a potential agonist (in comparison with PGE_1 alone). VASP phosphorylation in the presence of 15.6 μM Ap_4A (Fig. 3, hash-marked grey bar) was significantly reduced compared to that obtained with PGE_1 alone (Fig. 3, grey bar, $p < 0.01$), suggesting that Ap_4A acts as an agonist of $P2Y_{12}$. However, increasing the concentration of Ap_4A up to 250 μM did not further reduce PGE_1-stimulated VASP phosphorylation ($p > 0.05$ for the comparison among three Ap_4A concentrations tested), suggesting that Ap_4A is only a partial agonist of $P2Y_{12}$. Addition of the specific $P2Y_{12}$ antagonist AR-C69931 (10 μM) blocked Ap_4A-induced reduction of VASP phosphorylation (data not shown), indicating that this agonist effect of Ap_4A is mediated through $P2Y_{12}$. HPLC analysis of Ap_4A incubated with whole blood under the same conditions used in the VASP assay for up to 10 min (*i.e.*, the incubation time of the VASP assay) showed no loss of Ap_4A (data not shown). Likewise, the normal degradation products of Ap_4A, AMP and ATP, were low and did not increase over time and ADP was not detected (data not shown). Thus, the agonist effect of Ap_4A on $P2Y_{12}$ did not appear to be mediated by a metabolite of Ap_4A.

In the presence of increasing concentrations of Ap_4A, there was a dose-dependent blockade of ADP's effect, resulting in higher levels of phosphorylated VASP (Fig. 3, hash-marked open bars). The calculated PRI for the inhibition of VASP phosphorylation induced by ADP relative to the maximal VASP phosphorylation without ADP was 89.5 ± 2.1%, 74.7 ± 1.0%, 61.5 ± 9.4%, and 48.9 ± 6.2% for 0, 15.6, 62.5, and 250 μM Ap_4A, respectively. Thus, the IC_{50} for Ap_4A inhibition of the ADP-induced, $P2Y_{12}$-mediated response was >250 μM.

Agonist and antagonist properties of Ap_4A on $P2Y_1$ receptors

ADP stimulation of $P2Y_1$ results in platelet shape change which can be measured by the change in transmittance in an aggregometer. Ap_4A inhibited 3 μM ADP-induced platelet shape change in a dose-dependent manner (Fig. 4A, estimated IC_{50} 200 μM). ADP stimulation of $P2Y_1$ also results in increases in cytosolic Ca^{2+} (Fig. 1). Therefore, we evaluated the effect of Ap_4A on the ADP-induced increase in cytoplasmic Ca^{2+}. In platelets prepared in the absence of apyrase, *i.e*. without preserving the activity of $P2X_1$, and without addition of extracellular Ca^{2+}, 3 μM ADP produced an approximately 3.5-fold increase in FLUO-4 fluorescence and this increase was inhibited by Ap_4A in a dose-dependent manner (Fig. 4B), with an IC_{50} of 40.8 ± 12.3 μM. That a higher concentration of Ap_4A was required for 50% inhibition ADP-stimulated platelet shape change (~200 μM) than was required for inhibition of ADP-stimulated increase in cytoplasmic Ca^{2+} from intracellular stores (~40 μM) is not surprising since platelet shape change is likely triggered by a low threshold increase in cytoplasmic Ca^{2+}, and thus would require an amount of Ap_4A that reduces the cytoplasmic Ca^{2+} increase by greater than 50%. Thus, as evidenced by two independent assays, platelet shape change and increase in cytosolic Ca^{2+}, Ap_4A is an antagonist of $P2Y_1$ receptors.

Under the same conditions, but in the absence of ADP, Ap_4A at concentrations up to 250 μM did not induce an increase in cytoplasmic Ca^{+2} as measured by the FLUO-4 fluorescence ratio in comparison with HEPES-saline vehicle control (1.18 ± 0.09 vs. 1.09 ± 0.06, mean ± SEM, n = 5, p = 0.161). Thus, Ap_4A is not an agonist of $P2Y_1$.

Agonist and antagonist properties of Ap_4A on $P2X_1$ receptors

Platelet $P2X_1$ function was not a factor in the platelet aggregation, shape change, $P2Y_1$ release of internal Ca^{2+}, and $P2Y_{12}$-mediated VASP assays described above since activity of $P2X_1$ was not preserved under the conditions used to isolate platelets for those assays. However, when platelets were prepared using protocols to protect $P2X_1$ from desensitization and suspended in buffer containing 2 mM Ca^{2+}, Ap_4A at 0.1 μM and higher concentrations induced an increase in platelet intracellular Ca^{2+} (Fig. 5A). For Ap_4A concentrations between 0.1 and 10 μM, this increase was entirely due to the $P2X_1$-mediated entry of extracellular Ca^{2+}, because it was eliminated when platelets were resuspended in buffer containing EGTA instead of Ca^{2+} (Fig. 5). Moreover, failure of the $P2Y_1$ antagonist MRS2179 at 100 μM to inhibit the Ap_4A-stimulated rise in Ca^{2+} (Fig. 5A) indicated that this rise was not due to direct or indirect stimulation of $P2Y_1$. Similarly, the majority of the cytoplasmic Ca^{2+} rise produced by 50 μM Ap_4A was eliminated in the presence of EGTA and could not be blocked by MRS2179. However, there was a small, but statistically significant component of the Ca^{2+} increase at 50 μM Ap_4A that was not blocked by EGTA but was blocked by MRS2179, indicating some $P2Y_1$-mediated contribution at this high Ap_4A concentration. The biphasic dose-response observed for Ap_4A (Fig. 5A) was also observed with the specific $P2X_1$ receptor agonist β,γ-CH_2-ATP (data not shown). Finally, MRS2159, a selective $P2X_1$ antagonist [28], added in parallel with Ap_4A, dose-dependently blocked Ap_4A-stimulated Ca^{2+} entry into platelets (Fig. 6), demonstrating that the Ap_4A-induced rise in platelet cytoplasmic Ca^{2+} requires $P2X_1$. Taken together, these data show that Ap_4A is an agonist of platelet $P2X_1$ receptors.

When 50 μM Ap_4A was added to a submaximal concentration of a selective $P2X_1$ agonist, 20 μM β,γ-CH_2-ATP, the post-stimulation FLUO-4 MFI was higher than with 20 μM β,γ-CH_2-ATP alone (4.43 ± 0.20 *vs*. 3.57 ± 0.47, p = 0.034, n = 3). Thus, there was no evidence that Ap_4A is a $P2X_1$ antagonist, and the enhanced response suggested that Ap_4A augmented the $P2X_1$ agonist effect of 20 μM β,γ-CH_2-ATP.

Discussion

The major findings of this study are that Ap_4A, a known constituent of platelet dense granules, is: a) an antagonist of platelet $P2Y_1$ and $P2Y_{12}$ receptors, where it inhibits the effects of the agonist ADP, b) an agonist of $P2X_1$ receptors, and c) a partial agonist of $P2Y_{12}$ receptors. These stimulating and inhibiting activities are summarized in Table 1.

Antagonist effects of Ap_4A on platelet $P2Y_1$ and $P2Y_{12}$ receptors

Previously published studies showed that Ap_4A and its analogs inhibit ADP-induced platelet aggregation and several aspects of platelet activation including release action, cytoplasmic calcium mobilization, thromboxane production, fibrinogen binding, and platelet factor 3 activities[14]. This inhibition of platelet functions was considered to be mediated through the P_{2T} pathway[14], a poorly defined term used before the currently known purinergic receptors, $P2X_1$, $P2Y_1$ and $P2Y_{12}$, were cloned and their functions well characterized. In the current study, consistent with these previous reports [14], we found that Ap_4A inhibits 3 μM ADP-induced platelet aggregation. The platelet disaggregation that was observed with 100 μM Ap_4A (Fig. 2) suggests that Ap_4A inhibited the $P2Y_{12}$ pathway. On the other hand, we also found that Ap_4A inhibits 3 μM ADP-induced platelet shape change (Fig. 4A), which is mainly mediated by $P2Y_1$[1,25].

ADP-induced platelet signaling through $P2Y_1$ and $P2Y_{12}$ receptors can be examined by specific assays, i.e. ADP-induced calcium increase for $P2Y_1$ receptors and ADP-induced VASP phosphorylation decrease for $P2Y_{12}$ receptors (Fig. 1). In the present study, 3 μM ADP-induced platelet cytosolic Ca^{2+} rise was inhibited by Ap_4A with an IC_{50} of 40.8 μM (Fig. 4B). This result confirmed that Ap_4A is a $P2Y_1$ antagonist. In addition, the decrease in ADP-induced VASP phosphorylation was inhibited by Ap_4A in a dose-dependent manner (Fig. 3). While Ap_4A inhibition of ADP-stimulated, $P2Y_1$-mediated increase in cytosolic calcium was measured within seconds of adding Ap_4A and ADP to cells, the $P2Y_{12}$ mediated VASP response was measured 10 minutes after adding Ap_4A to whole blood, raising the possibility that metabolites of Ap_4A, and not Ap_4A *per se* may be responsible for effects on $P2Y_{12}$. However, this appears unlikely since HPLC analysis showed no breakdown of Ap_4A when incubated under VASP assay conditions. Taken together, these results indicate that Ap_4A is an antagonist for both $P2Y_1$ and $P2Y_{12}$ receptors. Such dual specificity on platelet $P2Y_1$ and $P2Y_{12}$ receptors has been recently reported for other compounds[29,30].

Although 3 μM ADP was used as the stimulant in all experiments, the IC_{50} for inhibition of platelet aggregation (9.8 ± 2.8 μM) was 4-fold lower than the IC_{50} for the inhibition of the $P2Y_1$-mediated increase of cytosolic Ca^{2+} (40.8 ± 12.3 μM) and more than 25-fold lower than the IC_{50} for the $P2Y_{12}$-mediated decrease in VASP phosphorylation (>250 μM). Our results are similar to the findings of Cattaneo *et al*. [29] who reported that both MRS2298 and MRS2496 inhibited ADP-induced platelet aggregation, shape change and $P2Y_1$-mediated Ca^{2+} increase but, at higher concentrations, these compounds also partially inhibited ADP-induced decrease of cAMP, possibly via the $P2Y_{12}$ pathway. Furthermore, Cattaneo *et al*. [29] reported that the IC_{50}s of MRS2298 and MRS2496 for inhibition of platelet aggregation were lower than those for cytosolic Ca^{2+} increase and shape change. These data suggest synergism between $P2Y_1$ and $P2Y_{12}$ antagonism. Indeed, the synergism of antagonism between $P2Y_1$ and $P2Y_{12}$ receptors using selective antagonists for each receptor has been well documented *in vitro*[31]. The present findings and those of Cattaneo *et al*.[29] demonstrate that synergism of inhibition may occur for a single compound with dual receptor antagonist properties. However, the present findings are the first report of an endogenous molecule released by platelets that has a dual and synergistic inhibitory effect on both $P2Y_1$ and $P2Y_{12}$ receptors. This synergism may be explained, at least in part, by the recently reported reciprocal cross-talk between $P2Y_1$ and $P2Y_{12}$ receptors[32,33].

Agonist effects of Ap_4A on platelet $P2Y_{12}$ but not $P2Y_1$ receptors

In addition to the inhibitory effects of Ap_4A on $P2Y_1$ and $P2Y_{12}$ pathways, we demonstrated that Ap_4A attenuated PGE1-stimulated VASP phosphorylation (Fig. 3). This attenuation was prevented by AR-C69931, demonstrating specificity for $P2Y_{12}$. These data indicates that Ap_4A either directly or indirectly (*e.g.*, via metabolites) results in platelet $P2Y_{12}$ activation. However, our study of metabolites after incubation with whole blood suggests such effects result directly from Ap_4A rather than its metabolites.

The apparently opposing effects of Ap_4A, as a weak agonist of $P2Y_{12}$, causing a decrease in VASP phosphorylation, and at the same time as a partial antagonist of $P2Y_{12}$, blocking ADP-stimulated decrease in VASP phosphorylation are both explained by a weak and inefficient interaction of Ap_4A with $P2Y_{12}$. When added alone, the effect of Ap_4A on VASP phosphorylation is maximal at 15.6 μM, suggesting saturation of Ap_4A binding to $P2Y_{12}$ at this Ap_4A concentration and higher. However, even with saturating concentrations of Ap_4A, only small decreases in VASP phosphorylation were observed, indicating the Ap_4A-$P2Y_{12}$ interaction triggers only a weak downstream signal. In contrast, ADP by itself causes a large decrease in VASP phosphorylation, suggesting efficient coupling of ADP-$P2Y_{12}$ with downstream events. Thus, the interaction of Ap_4A with $P2Y_{12}$, while causing weak downstream signals, prevents ADP from triggering stronger signals with larger decreases in VASP phosphorylation. The fact that very high concentrations of Ap_4A (250 μM) are required to block the ADP effect on VASP suggests that Ap_4A binding is reversible and that even binding of small amounts of ADP, as might occur over the 10 minute VASP assay incubation is sufficient to trigger a strong $P2Y_{12}$ coupled response.

Although Ap_4A and other diadenosine polyphosphates have been reported to activate $P2Y_1$ receptors in other cell types[34,35], their agonist activity on platelet $P2Y_1$ receptors is unknown. We found that Ap_4A stimulation did not result in $P2Y_1$-mediated increase of platelet cytosolic Ca^{2+} and, therefore, does not act as a platelet $P2Y_1$ receptor agonist.

Agonist effects of Ap_4A on platelet $P2X_1$ receptors

It is well known that Ap_4A is an agonist for P2X receptors in rat vas deferens and human urinary bladders[17,36]. In platelets, Sage *et al*.[4] showed that Ap_4A may induce a rise in cytosolic Ca^{2+} and suggested that this was mediated by $P2X_1$. Such an Ap_4A-induced platelet cytosolic Ca^{2+} rise was also observed in our study with extracellular Ca^{2+} available, but was obliterated when extracellular Ca^{2+} was removed by the chelating agent EGTA (Fig. 5A). These data confirm that the cytosolic Ca^{2+} increase resulted solely from an influx of extracellular Ca^{2+}, i.e. a P2X-mediated effect (Fig. 1). Moreover, MRS2159, a selective $P2X_1$ antagonist[28], blocked the Ap_4A-induced influx of extracellular Ca^{2+}, demonstrating Ap_4A is a platelet $P2X_1$ agonist. Although Ap_4A and other adenosine polyphosphates can be both agonists and antagonists of $P2Y_1$ and $P2Y_{12}$ receptors, the present study has demonstrated that Ap_4A functions only as an agonist, not an antagonist, for platelet $P2X_1$ receptors.

Possible pathophysiologic roles of Ap_4A

The plasma concentration of Ap_4A has been reported to range from 0.33 − 1.0 μM[8,10,13]. However, in the platelet-rich microenvironment where a thrombus is formed or high shear force is generated by vessel damage, the local concentration of Ap_4A may reach up to 100 μM as a result of dense granule release by involved platelets[8,10,13]. The present study has demonstrated that 0.1–1 μM Ap_4A has significant agonist effects on platelet $P2X_1$ receptors. In addition, Ap_4A inhibits ADP-induced aggregation and cytosolic Ca^{2+} increase with IC_{50}s of 9.8 ± 2.8 and 40.8 ± 12.3 μM, respectively. Although the concentrations of Ap_4A required to antagonize ADP effects on platelet $P2Y_1$ and $P2Y_{12}$ receptors are high compared to ADP, it is well known that Ap_4A and other diadenosine polyphosphates are more stable than ADP

and ATP[10]. Whereas ADP is rapidly inactivated by ecto-nucleotidase[37], Ap_4A is more resistant to hydrolysis[10] and therefore attains higher concentrations than ADP in plasma and in the local thrombotic microenvironment. Whether this high concentration can be reached in any pathophysiological conditions is unclear therefore the pathophysiologic role of high concentration Ap_4A is speculative.

Pharmacologic Perspectives

Ap_4A is the backbone of some chemical derivatives being developed as potential antiplatelet agents. *In vitro* antiplatelet[14,38] and *in vivo* antithrombotic effects[39,40] of Ap_4A and its derivatives have been reported, and antithrombotic potency is improved after certain chemical structure modifications[14,38]. The current study shows that Ap_4A has antagonist effects for platelet $P2Y_{12}$ receptors, the target of several effective antithrombotic agents including clopidogrel and prasugrel[41]. Ap_4A derivatives, while different in potencies and selectivity towards platelet receptors (results to be published), may have antiplatelet effects comparable to clinically available drugs such as clopidogrel. In clinical studies[5], standard doses of the widely-used, FDA-approved $P2Y_{12}$ antagonist clopidogrel reduced VASP PRI to 61.1 ± 17% mean ± SD) of maximal. In the present study, 62.5 μM Ap_4A resulted in a similar reduction of VASP PRI, to 61.5 ± 9.4%. Thus, Ap_4A released as a result of initial platelet activation may block subsequent activation of platelets to the same degree as pharmacological doses of clopidogrel. Furthermore, in the present study 250 μM Ap_4A reduced VASP PRI to 48.9 ± 6.2%.

The potential advantage of Ap_4A and its derivatives as therapeutic antiplatelet agents is that, unlike clopidogrel, prasugrel, or other $P2Y_{12}$ antagonist in development, they also have, as demonstrated in the present study, a synergistic inhibitory effect on platelet $P2Y_1$ receptors. One potential concern of using Ap_4A or its derivatives as antithrombotic agents might be their action on platelet $P2X_1$ receptors. Recent studies indicate that $P2X_1$ has an important role in platelet activation, particularly under conditions of shear stress[19]. Nevertheless, the present study on the mechanism of platelet inhibition by Ap_4A provides a framework for future development of Ap_4A-derived antiplatelet agents. Chemically modified Ap_4A derivatives may be compared to Ap_4A for their selective potency on platelet purinergic receptors.

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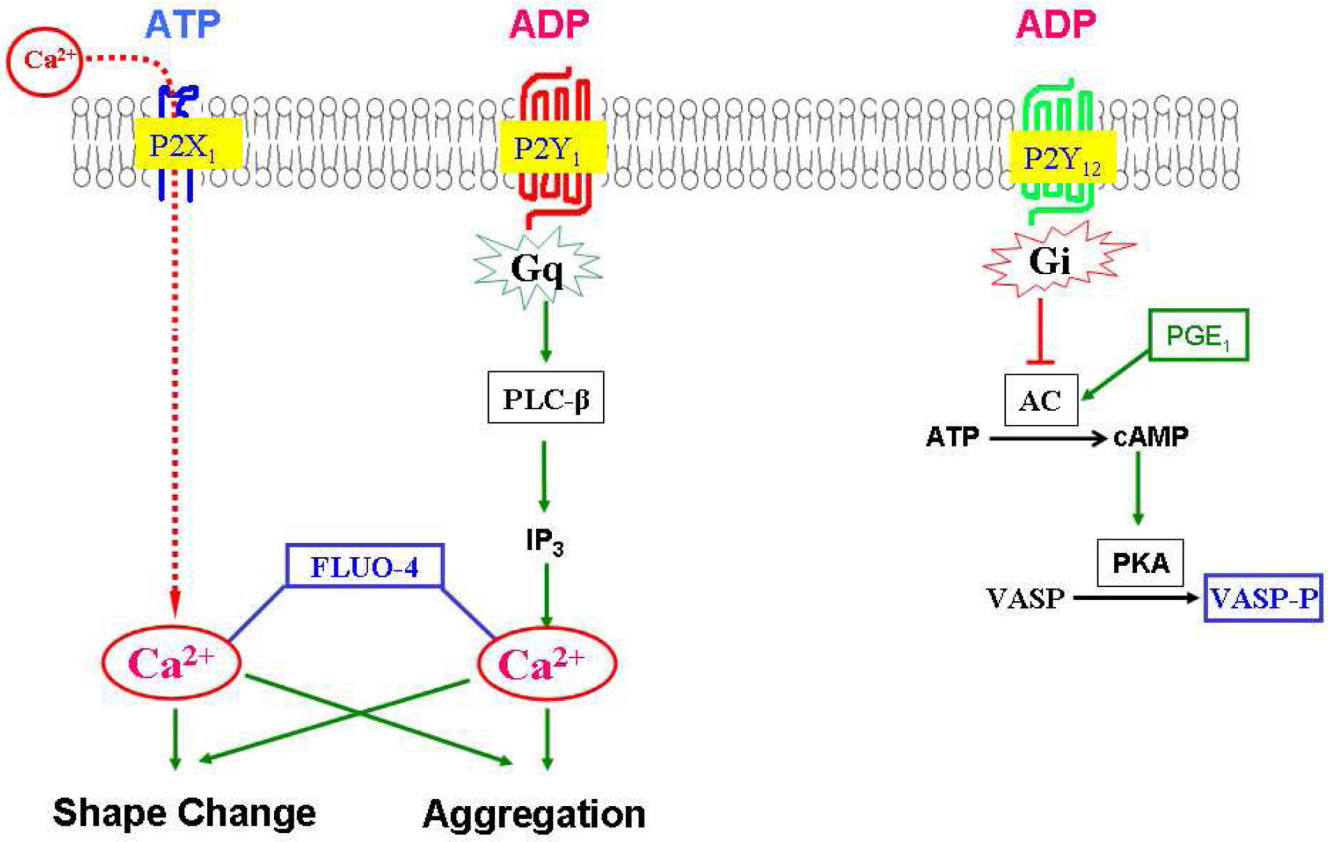

Figure 1. Platelets express three purinergic receptors, $P2Y_1$, $P2Y_{12}$ and $P2X_1$

The ligand is ADP for $P2Y_1$ and $P2Y_{12}$, and ATP for $P2X_1$. $P2X_1$ is a ligand-gated ion channel which allows extracellular Ca^{2+} to shift into the cytoplasm upon activation. ADP binding to the $P2Y_1$ receptor induces activation of phospholipase C β (PLC-β), which, via inositol triphosphate (IP_3), subsequently leads to release of cytoplasmic Ca^{2+} pools. Both $P2X_1$ and $P2Y_1$ activation cause cytosolic Ca^{2+} increase, which in the present study was measured by flow cytometry with the Ca^{2+} indicator, FLUO-4. ADP binding to platelet $P2Y_{12}$ receptors results in a decrease of cytoplasmic cAMP by inhibiting adenylyl cyclase. cAMP subsequently leads, via protein kinase A (PKA), to phosphorylation of vasodilator stimulated phosphoprotein (VASP), which was measured by flow cytometry.

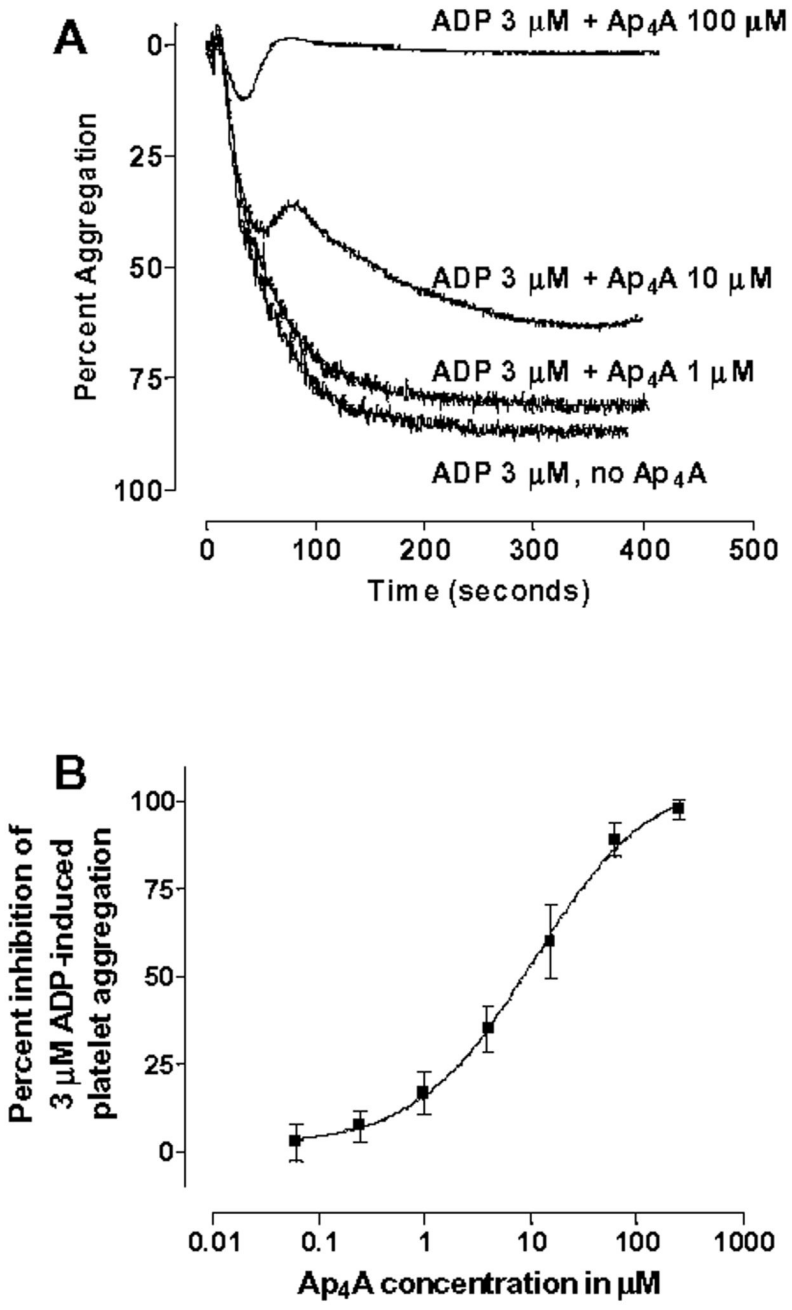

Figure 2. Ap_4A inhibits ADP-induced platelet aggregation

A, Platelet aggregation induced by 3 μM ADP was inhibited by Ap_4A in a dose-dependent manner. At 100 μM Ap_4A complete platelet disaggregation was observed. The experiments were performed in a Chrono-log® aggregometer. B, Dose response curve for Ap_4A inhibition of 3 μM ADP-induced platelet aggregation (mean ± SEM, n = 3). The results were obtained with the 96-well microplate reader method.

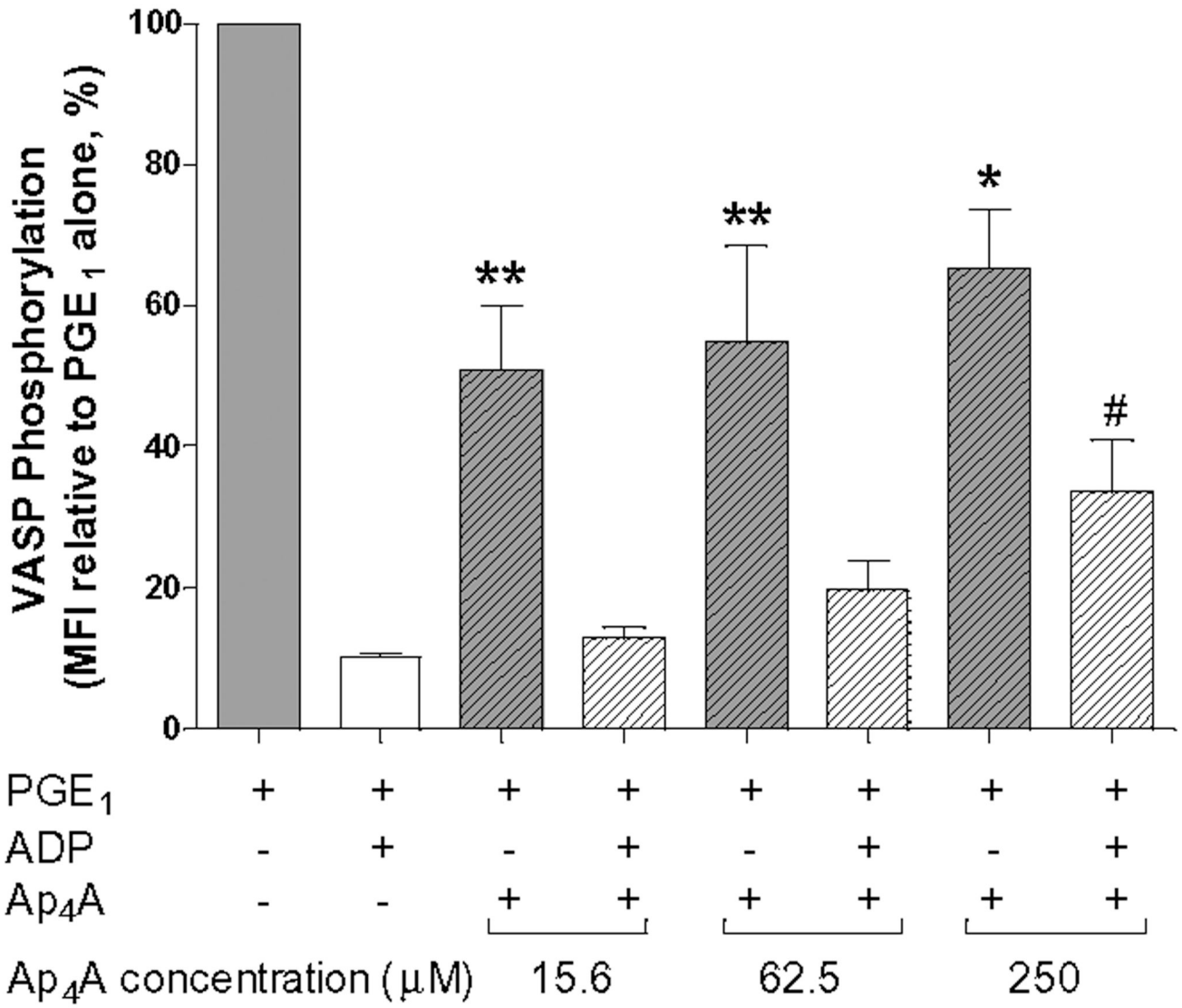

Figure 3. Ap_4A reduces VASP phosphorylation and attenuates the ADP-induced decrease in VASP phosphorylation

Platelets were treated with PGE_1 in every experiment, and the resultant MFI was used as unstimulated baseline (see Materials and Methods for details). As expected, the addition of 3 μM ADP reduced VASP phosphorylation (open bar). This ADP-induced decrease in VASP phosphorylation was attenuated by Ap_4A in a dose dependent manner (hash-marked open bars), # $p < 0.05$ compared with PGE_1 plus ADP (open bar). Ap_4A in the absence of ADP addition resulted in a decrease in VASP phosphorylation but not in a dose-dependent manner (hash-marked grey bars, * $p < 0.05$, ** $p < 0.01$ compared with PGE_1 alone. There was no statistically significant difference among the three tested concentrations of Ap_4A. The data (mean ± SEM, n = 3) were analyzed by one way ANOVA and Bonferroni's multiple comparison test.

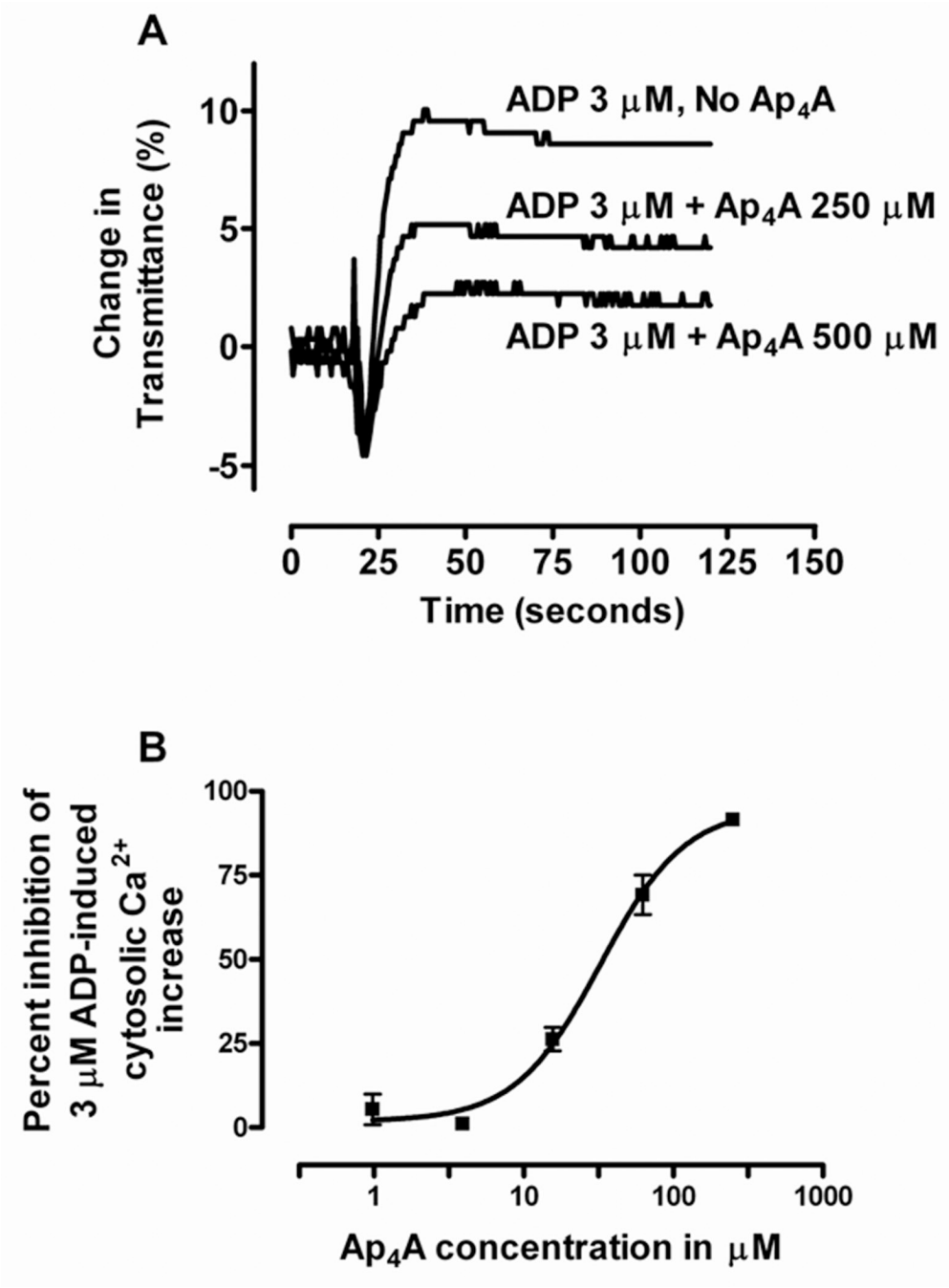

Figure 4. Ap_4A inhibits ADP-induced platelet shape change and cytosolic Ca^{2+} increase

A, Platelet shape change (upward deflection) induced by 3 μM ADP was inhibited by Ap_4A in a dose-dependent manner. Note that there was no platelet aggregation (downward deflection) after shape change because EDTA (10 mM) was mixed with PRP. Results shown are representative of 3 such experiments. B, Dose response curve of Ap_4A inhibition of 3 μM ADP-induced intracellular Ca^{2+} increase, measured by flow cytometry with the calcium indicator FLUO-4 (mean ± SEM, n = 4).

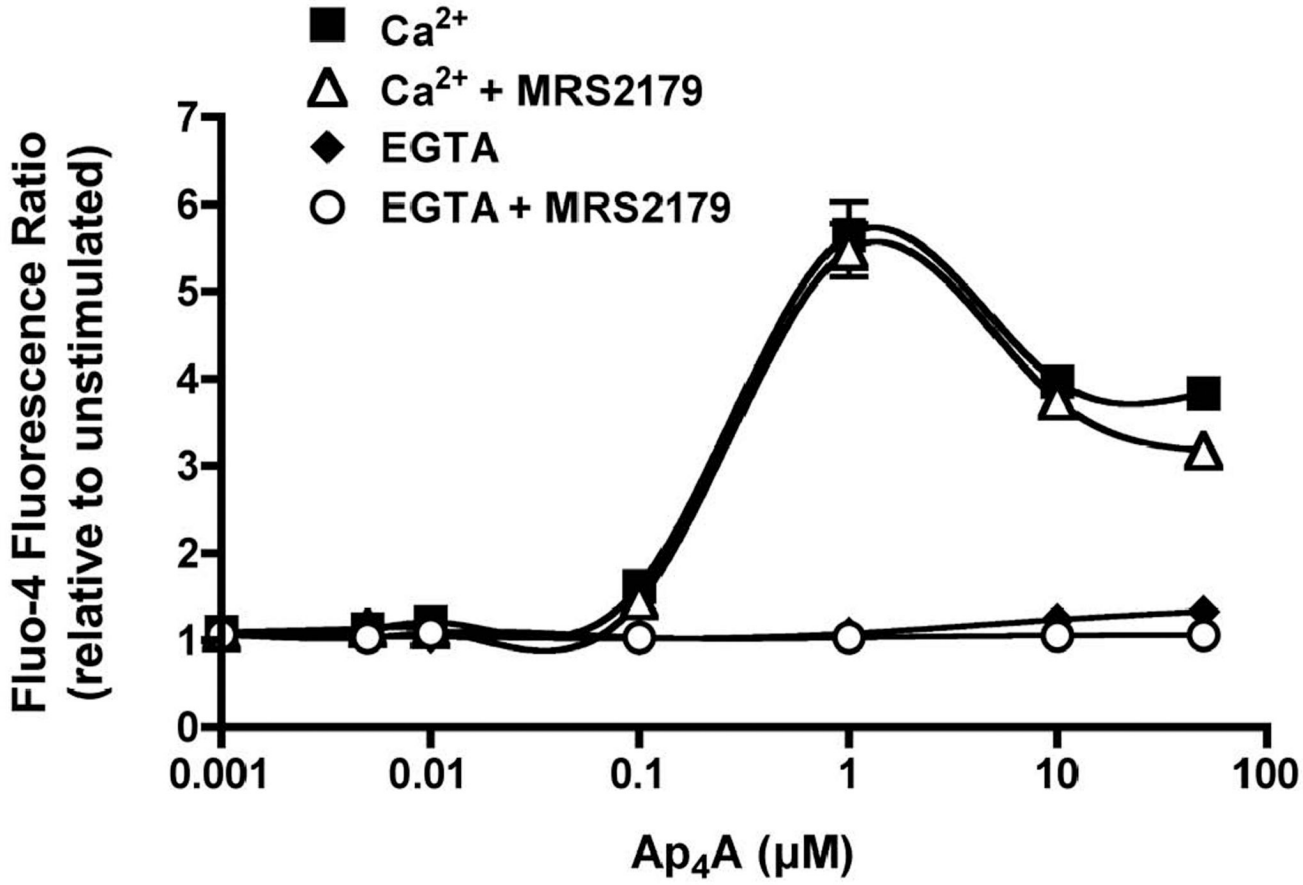

Figure 5. Dose response of Ap_4A-induced, $P2X_1$-mediated cytosolic Ca^{2+} increase. A

The experiments were performed under conditions specific for $P2X_1$ (see Materials and Methods for details), with the indicated buffers: (■) 2 mM Ca^{2+}; (♦) 1 mM EGTA, no Ca^{2+}; (△) 2 mM Ca^{2+} plus 100 μM MRS2179; (○) 1 mM EGTA plus 100 μM MRS2179, no Ca^{2+}. Results are mean ± SEM, n = 4.

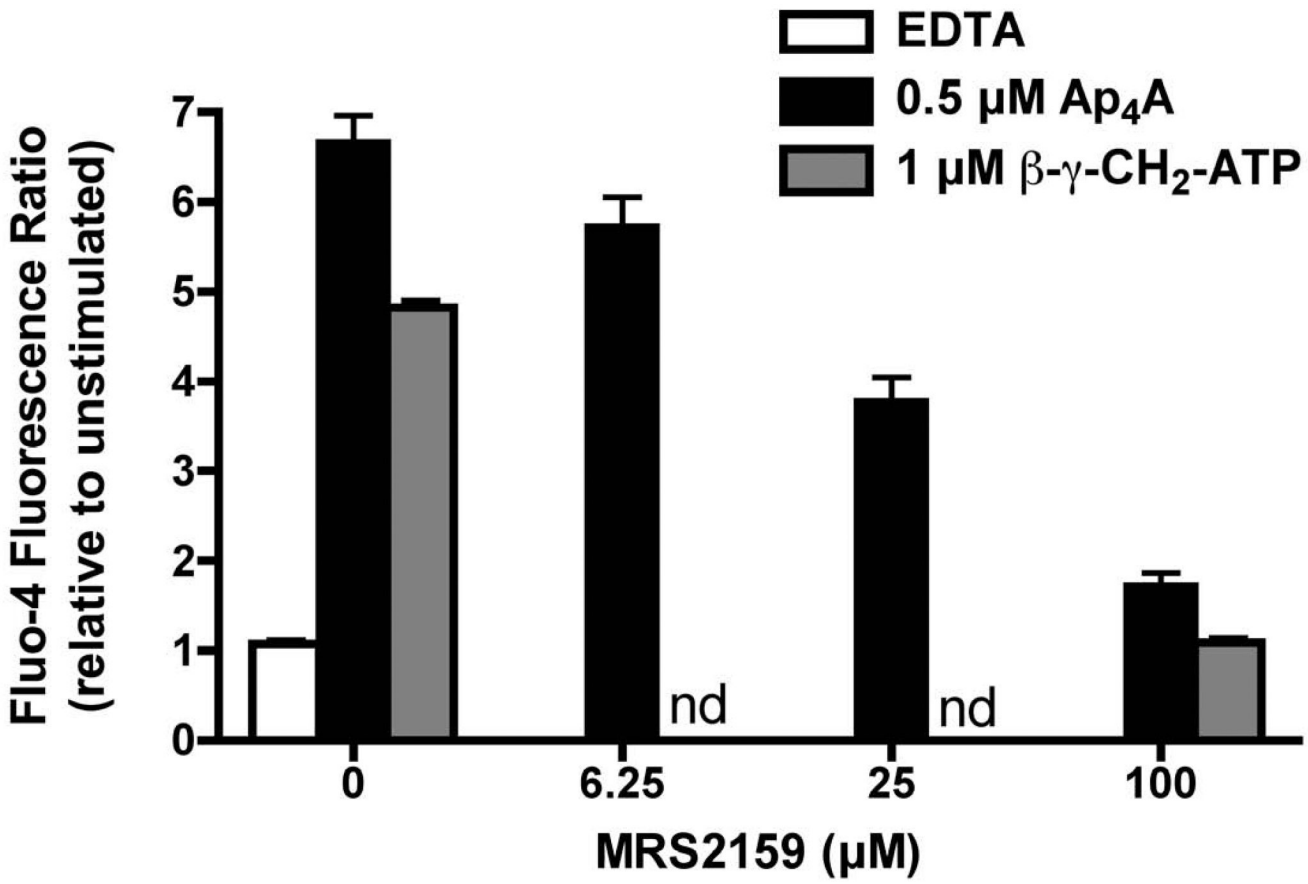

Figure 6. Dose-dependent MRS2159 inhibition of 0.5 μM Ap_4A- and 1 μM β,γ-CH_2-ADP-induced entry of extracellular Ca^{2+} into platelets

FLUO-4-loaded platelets suspended in 2 mM Ca^{2+} buffer were stimulated with either Ap_4A 0.5 μM (black bars) or 1 μM β,γ-CH_2-ATP (grey bars) in the presence of 0, 6.25, 25, or 100 μM MRS2159. Dependence on extracellular calcium is shown by results of control samples suspended in 2 mM EDTA without Ca^{2+} and stimulated with Ap_4A 0.5 μM (open bar). Results are mean ± SEM, n=3. Abbreviations: EDTA, ethylene diamine tetraacetic acid; nd, not done.

Table

Summary of Ap_4A platelet stimulating and inhibiting activities.

Assay	Ap_4A Agonist Activity	Ap_4A Antagonist Activity (inhibition of ADP or β,γ-CH_2-ATP)*
Aggregation ($P2Y_1$ and $P2Y_{12}$ mediated)	no activation at up to 250 μM	IC_{50} 9.8 ± 2.8 μM
Shape Change ($P2Y_1$ mediated)	no activation at up to 250 μM	IC_{50} ~200 μM
$P2Y_1$ release of internal Ca^{2+}	no activation at up to 100 μM	IC_{50} 40.8 ± 12.3
$P2Y_{12}$-mediated VASP	maximal at 15.6 μM	IC_{50} >250 μM
$P2X_1$ entry of external Ca^{2+}	EC_{50} ~0.3 μM (biphasic curve)	No inhibition at 50 μM

*Ap_4A antagonist activity was determined relative to 3 μM ADP stimulation of platelet aggregation, shape change, $P2Y_1$ release of internal Ca^{2+}, and $P2Y_{12}$-mediated VASP and relative to 20 μM β,γ-CH_2-ATP stimulation of $P2X_1$ entry of external Ca^{2+}.